ENVIRONMENTAL GROWTH REQUIREMENTS FOR SUBMERGED CULTURES OF THE ECTOMYCORRHIZAL FUNGUS *CENOCOCCUM GEOPHILUM* Fr.

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ABSTRACT — The ability of *Cenococcum geophilum* to produce an enlarged biomass of submerged mycelial condensations under different culture conditions was evaluated. Best production (mycelial dry weight 11,15 mg/ml) occurred in 10 l flasks of glucose di-ammonium tartrate casein medium (pH 5.8) flushed with 90 l / hour of sterile air and inoculated with 5% w-w of fresh mycelium with incubation in the light (100 lux) during 28 days at 25° C.

RÉSUMÉ — La capacité de *Cenococcum geophilum* à produire une biomasse considérable de mycélium submergé dans différentes conditions de culture a été testée. Le meilleur rendement (poids sec de mycélium 11,15 mg/ml) a été obtenu dans des flacons de 10 litres, contenant un milieu glucose, di-ammonium tartrate et caséine (pH 5.8), dans lesquels 90 litres d'air stérile sont injectés par heure. Ces flacons sont inoculés avec 5% poids/poids de mycélium frais et incubés 28 jours sous éclairage (100 lux) à 25° C.

INTRODUCTION

Ectomycorrhizal fungi occur in nearly all soils on earth and form a symbiotic relationship with roots of most terrestrial plants.

Mycorrhizal fungi are known to benefit plant growth in several ways as nutrient uptake (Harley & Smith, 1983) and in pathways involving phosphorus nutrition (Morton *et al.*, 1990). These fungi increase plant tolerance to ■ number of stressors or activate transport of water to the roots (Parke *et al.*, 1983). In addition, soil or seedling inoculation with ectomycorrhizal fungi make possible the introduction of tree plants in areas which would not otherwise support their growth (Harvey, 1991).

The first step in an artificial inoculation program requires the development of appropriate iinocula. There is a diversity of sources of mycorrhizal inocula and few patent applications have been made with most involving particular growth substrates (Menge *et al.*, 1978; Marx *et al.*, 1981; Baltruschat & Dehne, 1985; Wood *et al.*, 1985).

In a previous paper (Job & Aragno, 1992) we investigated nutritional growth requeriments of *Cenoccum geophilum* in submerged cultures. This fungus has been frequently reported in ectomycorrhizal associations with several economically important tree families (Heslin & Douglas, 1986; Danielson & Pruden, 1989).

In the present work, we investigated the ability of this taxon to produce large amounts of mycelial pellets under a range of different cultural conditions. We can thus propose an optimized culture environment with the final objective of producing sufficient quantity of inocula for large-scale forest application.

MATERIALS AND METHODS

Strain

Experiments were conducted with strain XO1-20) supplied by Vitroculture SA., Porrentruy, Switzerland; isolation locality is Birmensdorf, Zurich.

Preparation of mycelial inoculum

Cultures of *Cenoccum geophilum* were grown in Gd-ATC (glucose di-ammonium tartrate casein medium: Job & Aragno, 1992) at 25° C. After an incubation period of 21 days in dark conditions, mycelial pellets were aseptically homogenized and used as inocula for all experiments.

Culture media

The selected strain was grown in Gd-ATC. For each test, five 10 l flasks containing 7 l of sterile medium (sterilized 90 min at 121° C) were used.

At the end of the incubation period (28 days), mycelial pellets were washed and dried. Fungal biomass is expressed as mg dry weight/ ml culture medium with given values being means of five replicates.

Temperature and light intensity

Flasks (pH 5) inoculated with 3% of inoculum were incubated 28 days (air flushing 60 l/h) at 15, 25 or 30° C and 100, 200, 300 lux 12 hs/day or in complete darkness.

Air flushing

Flasks (pH 5) inoculated with 3% of inoculum were flushed with 10, 30, 60, 90 and 120 l/h sterile air and incubated 28 days at 25° C under 100 lux at 12 hs/day.

pH

Flasks inoculated with 3 % of inoculum, flushed with 90 l/h sterile air, with pH adjusted (0.1 M HCl or NaOH) to 2.2, 3.4, 4.6, 5.8 and 7.0 were incubated 28 days at 25° C and under 100 lux at 12 hs/day.

Size of inoculum

Flasks (pH 5.8) were inoculated with 7 differents levels of inocula (1%, 2%, 3%, 4%, 5%, 7.5% and 10% w-w) and then incubated 28 days at 25° C, under 100 lux at 12 hs/day and flushed with 90 l/h sterile air.

T°C	LUX			
	0	100	200	300
15	2.12 +/- 0.12	4.15 +/- 0.17	4.28 +/- 0.26	4.31 +/- 0.21
25	3.96 +/- 0.21	6.60 +/- 0.27	6.53 +/- 0.35	6.27 +/- 0.40
30	4.12 +/- 0.14	6.27 +/- 0.19	6.61 +/- 0.38	6.07 +/- 0.35

Table 1. — Mycelial dry weight (mg/ml) of *Cenococcum geophilum* (mean of 5 replicates) obtained in the different conditions of light and temperature tested.

Tableau 1. — Poids sec du mycelium de *Cenococcum geophilum* (moyenne de 5 expériences) obtenu dans différentes conditions de lumière et températures.

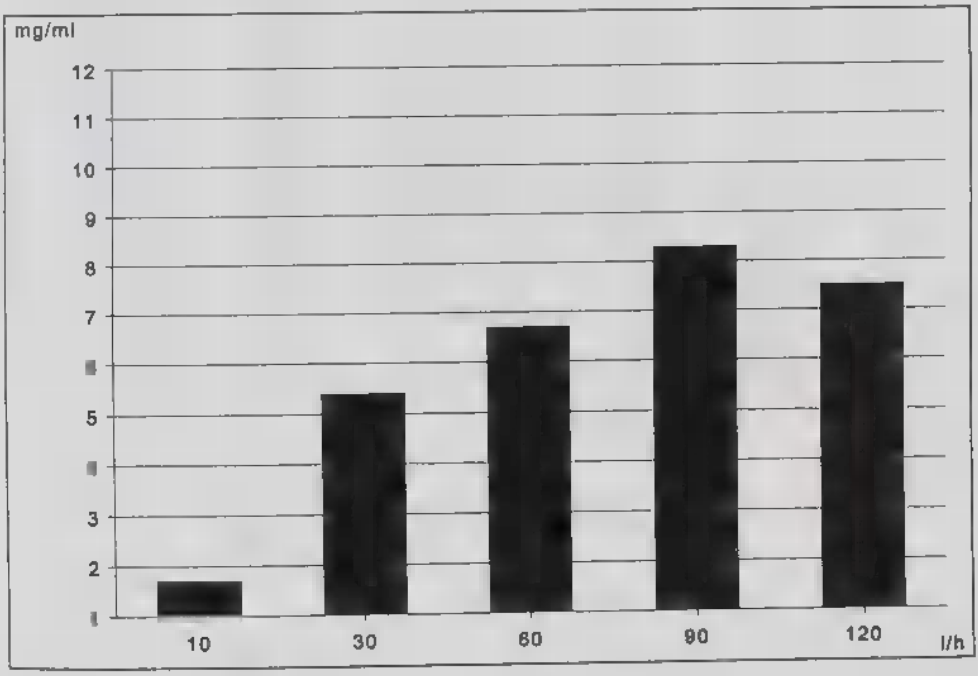

Fig. 1 : Mycelial biomass (mg dry weigt/ml) obtained after 28 days, in Gd-ATC medium with different air flush (l/h).

Fig. 1: Biomasse du mycélium (mg poids sec/ml) produite après 28 jours, dans un milieu Gd-ATC avec différent pompage d'air stérile (l/h).

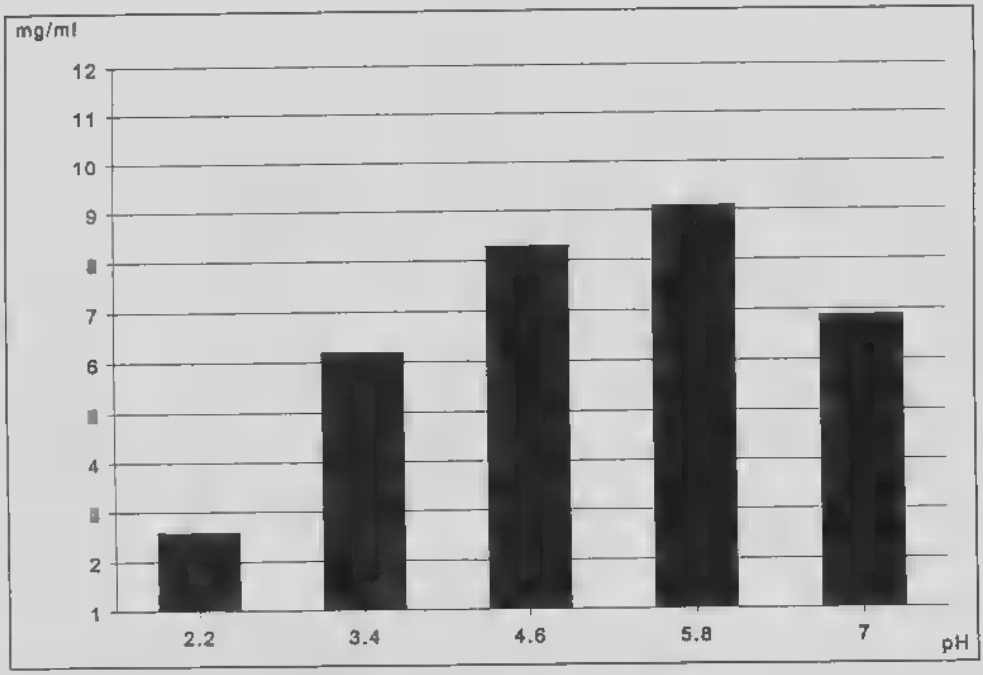

Fig. 2: Mycelial biomass (mg dry weigt/ml) obtained after 28 days, in Gd-ATC medium with different pH.

Fig. 2: Biomasse du mycélium (mg poids sec/ml) produite après 28 jours, dans un milieu Gd-ATC avec différent pH.

RESULTS

Table I shows yields as mycelium dry weight mg/ml culture medium obtained under different conditions of light and temperature.

Both temperature and light are important for the mycelial growth of filamentous fungi. Present data indicate dark conditions were unsuitable for growth of *Cenoccum geophilum*. Indeed, table I shows, that a maximum growth at 25 or 30° C was obtained only under light conditions. However no significant yield increment was obtained with increase in light ntensity from 100 to 300 lux.

Figures 1, 2 and 3 relate mycelial growth to the amount of air flushing, inoculum size or to pH.

We observed, (figure 1) that a flush of 90 liters of sterile air per hour was the optimum to support best mycelial growth. A decrease of this flush considerabily reduced mycelial yield and at a flush of 10 l/hs the fungus failed to grow vigorusly.

A similar result appear from figures 2 or 3. A neutral pH or a too acid value (2.2 or 3.4) affected fungal yield production of *C. geophilum*. An inoculum level of 5% w-w proved to be the optimum to support a good mycelial growth. A decrease in the amount of the inoculum also negatively affected fungal production.

Beside, figure 4 compares growth curves of *C. geophilum* developing in a standard media for ectomycorrhizal fungi as Melin-Norkans' modified medium: MMN (Job et Aragno, 1992) and in an optimized medium for *C. geophilum* (Gd-ATC, pH 5) in standard culture conditions (Job et Aragno, op.cit) with growth data of the same fungus in Gd-ATC medium at pH 5.8 but inoculated with 5% of mycelia and incubated in the optimized conditions (25° C; 100 lux 12 hs/day and flushed with 90 l sterile air /hs).

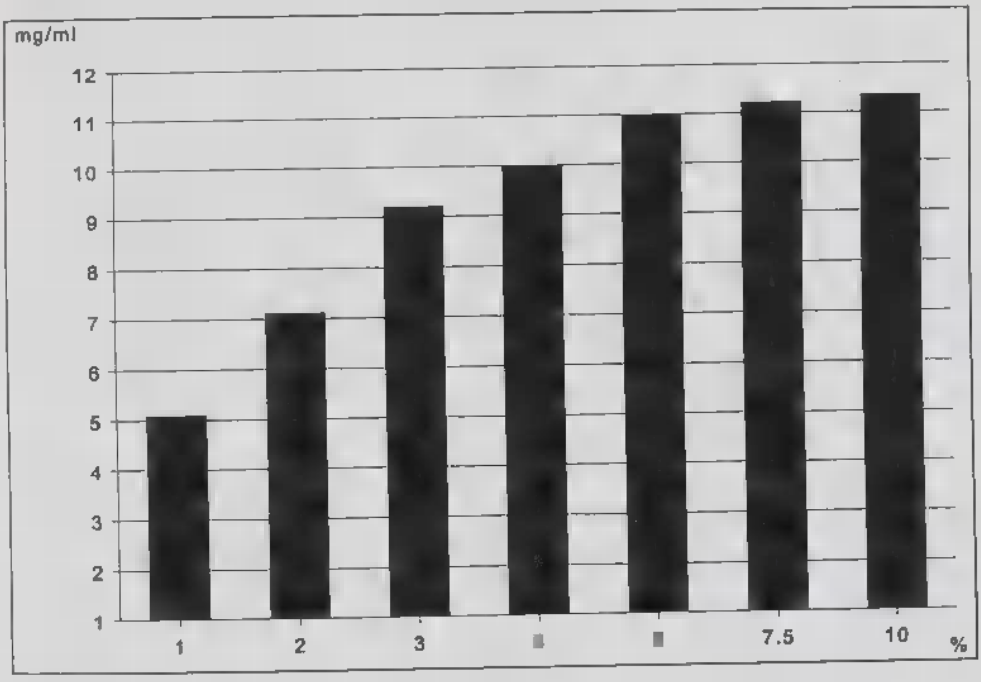

Fig. 3: Mycelial biomass (mg dry weigt/ml) obtained after 28 days, in Gd-ATC medium inoculated with 7 differents levels of inoculum.

Fig. 3: Biomasse du mycélium (mg poids sec/ml) produite après 28 jours, dans un milieu Gd-ATC inoculé avec 7 différentes quantites d'inoculum.

Beside, figure 4 compares growth curves of *C. geophilum* developing in a standard media for ectomycorrhizal fungi as Melin-Norkans' modified medium: MMN (Job et Aragno, 1992) and in an optimized medium for *C. geophilum* (Gd-ATC, pH 5) in standard culture conditions (Job et Aragno, op.cit) with growth data of the same fungus in Gd-ATC medium at pH 5.8 but inoculated with 5% of mycelia and incubated in the

DISCUSSION

Ectomycorrhizal fungi exhibit a wide range of pH tolerance (pH 3.0-7.0). However, in vitro, pH affects the growth in culture of several ectomycorrhizal fugi (Suvercha *et al.* 1991)

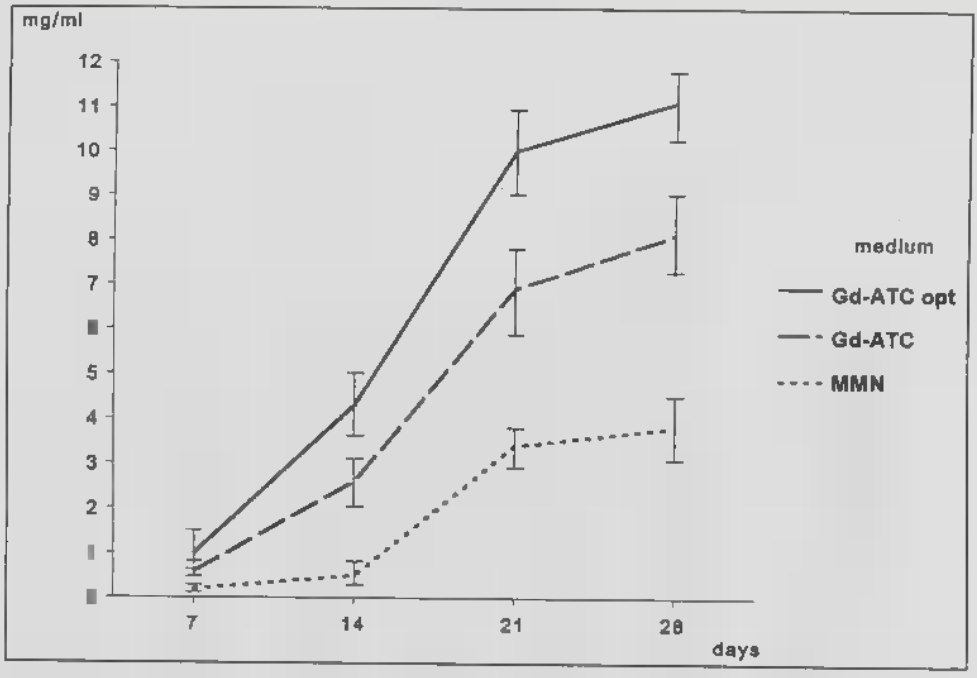

Fig. 4: Growth curves of *Cenococcum geophilum* (mg dry weight/ml) in two media for ectomycorrhizal fungi (MMN and Gd-ATC) in standard conditions and in the Gd-ATC in the optimized conditions.

Fig. 4: Courbes de croissance de *Cenococcum geophilum* (mg poids sec/ml) dans deux milieux pour champignons ectomycorrhiziques (MMN et Gd-ATC) en conditiones standard et dans le milieu Gd-ATC en conditions optimales.

Our work showed that different values of temperature and pH exerted significant effects on the mycelial growth of *C. geophilum*. We found that the temperature and pH required for optimum growth in vitro of *C. geophilum* were similar to those required for other fungi (Song *et al.* 1987). However, in comparison to other ectomycorrhizal fungi, *C. geophilum*, requires a relatively high light level for optimum submerged mycelial condensations growth.

We also observed the increment of growth of this fungus when we increased the air flush, showing that mycelium growth used oxygen and an adequate supply of this is an essential requirement throughout the fermentation cycle. One of the advantages of the air flush design here introduced, was the use of a sparger which admits a stream of small air bubbles resulting in a more effective aeration and smaller pellets as compared to pellets obtained using mechanical agitation (Job et Aragno, 1992).

Therefore, a well-balanced aeration and agitation system is necessary, due to the partialy contradictory requirements of filamentous fungi: the necessity of high oxygen tension and a high sensitivity against mechanical stress (figure 1 shows that a very high flush decreased the mycelial growth).

Contrary to the general idea that ectomycorrhizal fungi grow very slowly in

liquid media (Chapman et al., 1990) our results stress that optimization of the culture medium and environmental conditions, allows fast growth developing a marked biomass in submerged cultures. Such is indicated by the high biomass yield developed by *Cenococcum geophilum* to levels approximatring yields developed by several saprophytic fungi (Song et al., 19 87).

ACKNOWLEDGEMENTS

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